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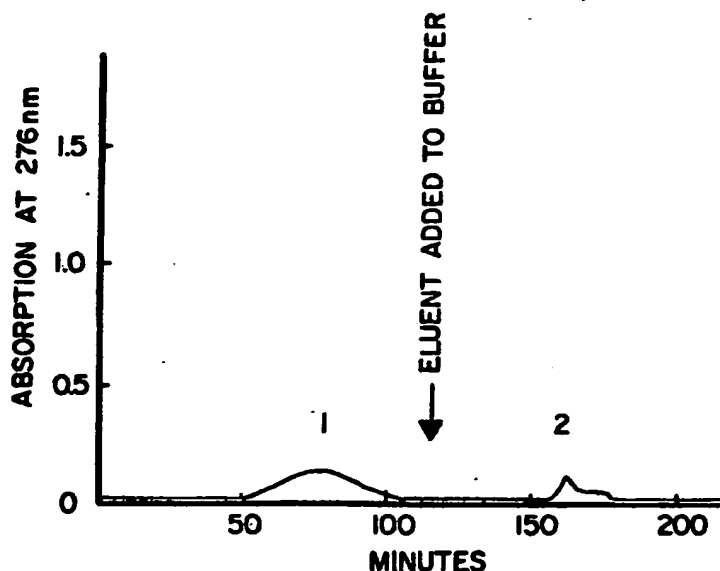
(57) Abstract

Synthesized succinyl and glutaryl glucosamines, p-(succinylamido)-phenyl-α-D-gluco- and mannopyranosides, p-(glutarylamido)-phhenyl-α-D-gluco- and mannopyranosides and p-(isothiocyanotophenyl)-α-D-gluco- and mannopyranosides are reacted with insulin to form corresponding glycosylated insulins containing from 1 to 3 glycosyl groups per insulin molecule. The novel glycosylated insulins resist aggregation and show significant activity in depressing blood sugar levels.

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GLYCOSYLATED INSULIN DERIVATIVES

Background of the Invention

This invention relates to the preparation of glycosylated insulins. More particularly, this invention relates to the preparation of glycosylated insulins and to novel intermediates to be used in preparing glycosylated insulins.

Various systems have been proposed for the delivery of insulin to a diabetic patient that will be more responsive to the needs of the patient.

The bioengineering approach is directed towards design of insulin infusion pumps. Hundreds of diabetics presently use external battery-operated pumps. The pump injects insulin continuously through a needle attached to a catheter inserted into a vein or into subcutaneous tissue. The flow can be adjusted manually when a change occurs in the amount of insulin needed. The units are usually worn on a belt or strapped to a leg.

Still in an experimental stage are pumps that deliver an amount of insulin precisely determined by a sensor that measures blood glucose levels. Though successful progress has been made in this area, these pumps are still too heavy to be portable. Another difficulty is that the system needs an apparatus for the continuous sampling of blood, an analyzer to determine the blood glucose level rapidly and continuously, a computer to analyze the results and to determine the appropriate insulin dose, and an infusion pump to deliver insulin intravenously in a manner approximating the delivery by the beta cells of the pancreas. Efforts are underway to reduce the size of the system and prolong its sensor's life. A "vest pocket" model, a system the size

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1. of a cigarette pack containing glucose sensor, power source, computer, insulin reservoir and pump, has been reported by Elliot in _J. Am. Med. Assoc._, 241, 223 (1979).

5   Another obstacle at present is the lack of an accurate implantable electrode to sense the concentration of blood glucose. Again, a through-the-skin connection to the patient's blood stream for long periods presents risks of infection and clotting problems. Also, the

10   occurring aggregation of insulin in the artificial delivery systems poses a considerable problem since the aggregated insulin will precipitate or crystallize out of solution, thereby reducing the bioavailability of the insulin in an insulin reservoir. In addition, the

15   aggregated insulin can become lodged in the delivery needle and prevent the flow of insulin from the delivery system to the diabetic.

Objects and Summary of the Invention

It is, therefore, an object of the present invention

20   to prepare a semisynthetic insulin which will not aggregate as rapidly as native insulin and, therefore, have a longer storage life.

It is also an object of the present invention to prepare novel intermediate compounds to be used in the

25   preparation of non-aggregating semisynthetic insulins.

A still further object of the present invention is to prepare glycosylated insulins which possess significant biological activity in depressing blood sugar levels.

30   These and other objects may be accomplished by means of novel glycosylated insulins having one of the general formulae:
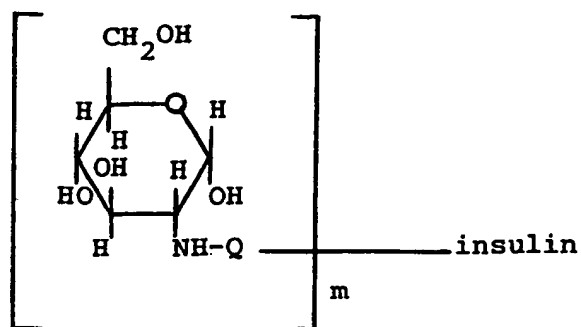
1

5

$$
\left[
\begin{array}{c}
CH_2OH \\
\text{H} \quad \text{O} \quad \text{H} \\
\text{H} \\
\text{OH} \quad \text{H} \\
\text{HO} \qquad \text{OH} \\
\text{H} \quad \text{NH-Q}
\end{array}
\right]_m
\text{—insulin}
$$

10

15

$$
\left[
\begin{array}{c}
CH_2OH \\
\text{H} \quad \text{O} \\
\text{H} \\
\text{OH} \quad Z \\
\text{HO} \\
\text{H} \quad X
\end{array}
O\text{—}\text{—NH-Q}
\right]_m
\text{—insulin} \quad \text{and}
$$

20

25

$$
\left[
\begin{array}{c}
CH_2OH \\
\text{H} \quad \text{O} \\
\text{OH} \quad Z \\
\text{HO} \\
\text{H} \quad X
\end{array}
O\text{—}\text{—N-C-}
\right]_m
\text{—insulin}
$$

with S above C (double bond) in the last structure.
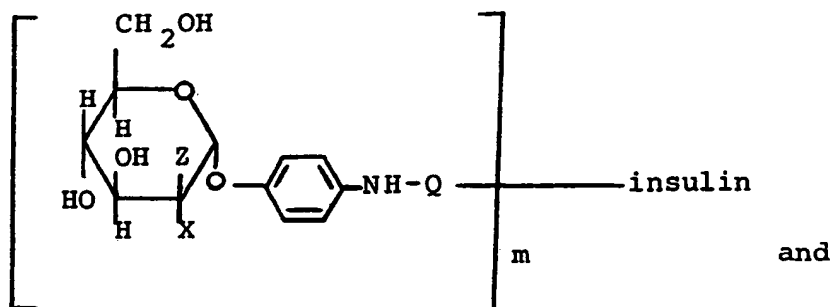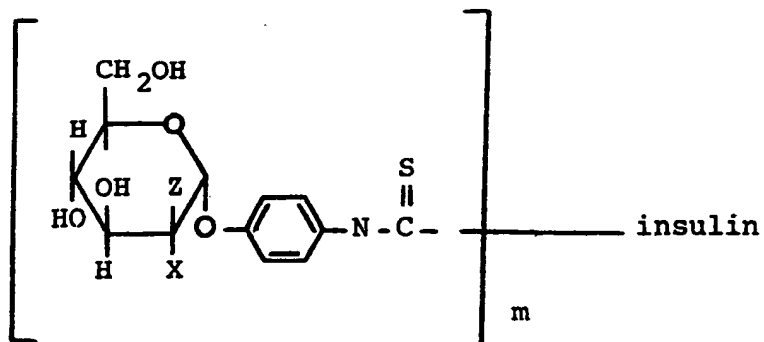
30

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1   where m is an integer of 1 to 3, X and Z are different
     and are selected from the group consisting of -H and -OH
     and -Q- is a dicarboxylic acid spacer group having the
     formula:

$$-\overset{O}{\overset{\|}{C}}-(CH_2)_n-\overset{O}{\overset{\|}{C}}-$$

5   where n is an integer of from 2 to 6 and is preferably 2
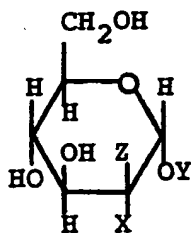   or 3.

Brief Description of the Drawings

     Figs. 1 through 4 of the drawings show elution
10   profiles from chromatography tests as reported and
     further described in examples IX, X, XI and XII,
     respectively.

Detailed Description of the Invention

     It is known that insulin can be combined with
15   maltose as taught by Brownlee et al, in _Science_, 206,
     223 (1979). However, this derivative of a disaccharide
     and insulin has been found not to possess any significant
     bioactivity in depressing blood sugar levels.

     In the present invention, the intermediates prepared
20   for coupling with insulin all consist of a glucose or
     mannose monosaccharide coupled to a spacer group. The
     spacer groups are derived from dicarboxylic acids, acid
     anhydrides or phenyl amines or a combination thereof. The
     intermediates have the following general formula:

25

30

$$\begin{array}{c} CH_2OH \\ \end{array}$$

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wherein Y is a member selected from the group

consisting of H, $-\langle\bigcirc\rangle-NH\overset{O}{\overset{\|}{C}}-(CH_2)-_n\overset{O}{\overset{\|}{C}}OH$ or $-\langle\bigcirc\rangle-N=C=S;$

X is a member selected from the group consiting of

$-H$, $-OH$ or $-NH\overset{O}{\overset{\|}{C}}-(CH_2)-\overset{O}{\overset{\|}{C}}OH;$ and

Z is a member of the group consisting of $-H$ or $-OH$,

with the proviso that when Y is $-H$, Z must also

be $-H$ and X must be $-NH\overset{O}{\overset{\|}{C}}(CH_2)_{\overline{n}}\overset{O}{\overset{\|}{C}}OH,$

when X is $-OH$, Z must be $-H$ and Y must be

$-\langle\bigcirc\rangle-NH-\overset{O}{\overset{\|}{C}}-(CH_2)_{\overline{n}}\overset{O}{\overset{\|}{C}}OH$ or $-\langle\bigcirc\rangle-N=C=S,$ and

when Z is $-OH$, X must be $-H$ and Y must be

$-\langle\bigcirc\rangle-NH\overset{O}{\overset{\|}{C}}(CH_2)_{\overline{n}}\overset{O}{\overset{\|}{C}}OH$ or $-\langle\bigcirc\rangle-N=C=S;$

and where n is an integer of 2 to 6.

Preferably n is an integer of 2 or 3 and the

$-\overset{O}{\overset{\|}{C}}(CH_2)_n-\overset{O}{\overset{\|}{C}}$ portion of the spacer is derived from succinic

or glutaric anhydride.

The intermediates described by the above formula may
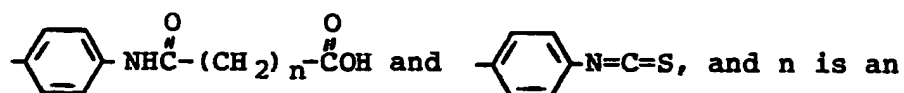be broken down into two subgroups.

The first subgroup is the glucosamine derivatives

wherein Z is $-H$, Y is $-H$ and X is $-NH\overset{O}{\overset{\|}{C}}-(CH_2)_n-\overset{O}{\overset{\|}{C}}OH,$
wherein n is an integer of 2 to 6.

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1   The second subgroup is the N-succinyl or
        N-glutaryl-amido-phenyl-α-D-gluco- and mannopyranosides
        and the p-isothiocyanotophenyl-α-D-gluco- and
        mannopyranosides wherein X and Z are different and are
5   selected from the group consisting of -H and -OH and Y is
        a member selected from the group consisting of

$$\text{—}\langle\text{phenyl}\rangle\text{—NHC(=O)-(CH}_2)_n\text{-COH}\quad\text{and}\quad\text{—}\langle\text{phenyl}\rangle\text{—N=C=S,}\quad\text{and } n \text{ is an}$$

10   integer of 2 to 6.
             The starting materials for the preparation of the
        sugar plus spacer glycocylated intermediates are
        glucosamine and p-nitrophenyl-α-D-gluco- and
        mannopyranosides and are commercially available.
15   The glucosamine may be reacted directly with an acid
        anhydride. Since the preferred spacers are succinyl and
        glutaryl moieties, the remainder of the discussion will
        be directed toward these derivatives. However, by
        appropriate synthesis, the 4 corresponding derivatives
20   from adipic, pimelic and suberic acids may also be
        utilized.
             The p-nitrophenyl-α-D-gluco- and mannopyranosides
        are first treated to reduce the nitro group to an amino
        group. They may then be reacted with succinic and
25   glutaric anhydrides to produce the corresponding
        N-succinyl- and N-glutaryl derivatives.
             The p-aminophenyl-α-D-gluco- and mannopyranosides
        may also be reacted with thiophosgene to form the
        corresponding p-isothiocyanotophenyl-α-D-gluco- and
30   mannopyranosides. The synthesis of these products are
        detailed in the examples which follow.

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The glycosylated intermediates which follow are representative of the novel pyranosides which may be used to couple with insulin.

$$NHC-(CH_2)_n-COH$$
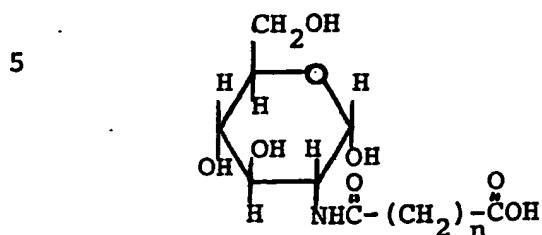
n=2   N-succinyl glucosamine   mp   174-175° C

n=3   N-glutaryl glucosamine   mp   195-196° C

$$-O-\phenyl-NHC-(CH_2)_n COH$$
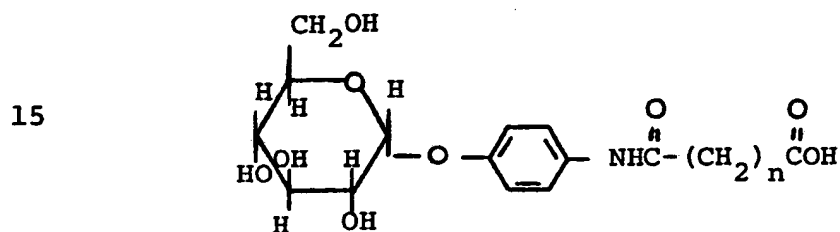
n=2   p-(succinylamido)-phenyl-α-D-glucopyranoside
         mp   178-180° C

n=3   p-(glutarylamido)-phenyl-α-D-glucopyranoside
         mp   167-168° C

$$-O-\phenyl-NHC-(CH_2)_n-COH$$
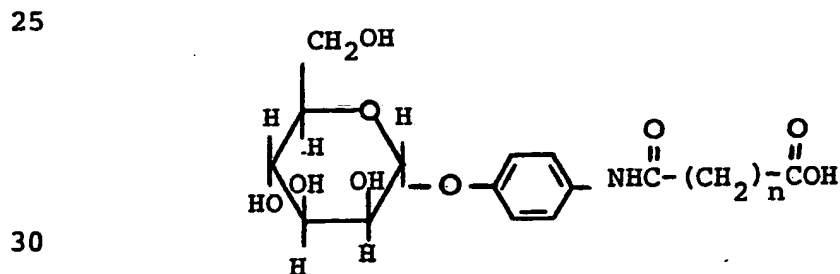
-8-

1   n=2   p-(succinylamido)-phenyl-α-D-mannopyranoside
                mp   65-66° C

       n=3   p-(glutarylamido)-phenyl-α-D-mannopyranoside
                mp   134-136° C
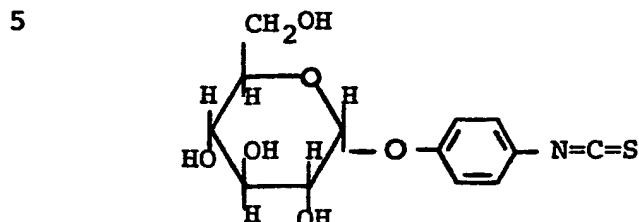
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10

p-isothiocyanotophenyl-α-D-glucopyranoside

15
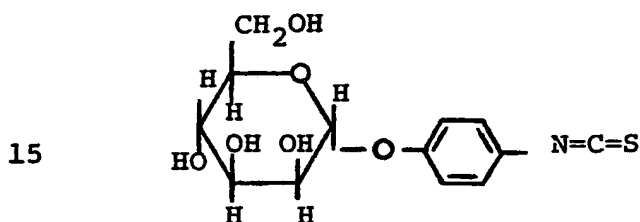
p-isothiocyanotophenyl-α-D-mannopyranoside

        The structure of the insulin molecule is well known.
20 It consists of two polypeptide chains A and B linked
    together by disulfide bonds of cystine. The N terminal
    group of the A fraction is glycine (Gly A-1) and the
    N-terminal group of the B fraction is phenylalanine (Phe
    B-1). Both N-terminal positions contain reactive free
25 α-amino groups. Adjacent the C-terminal group of the B
    fraction is lysine having a free ε-amino group. It is
    believed that these free amino groups contribute to the
    problem of aggregation of insulin molecules with their
    eventual precipitation.
30   By blocking these groups with the above glycosylated
    intermediates, it was believed that the bioactivity of

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1 the insulin would not be greatly affected and that
aggregation could be significantly inhibited or
prevented. In addition, it is believed that glycosylated
insulins may have other properties which may contribute
5 to a chemical-sustained release mechanism for delivery of
insulin to a diabetic in direct response to a change in
blood sugar levels without the need for external or
implanted sensing devices.

  The reaction of the intermediates shown above was
10 carried out by conversion of the carboxylic acid at the
end of the spacer to a mixed anhydride through reaction
with an alkylchloroformate and reaction of the mixed
anhydride with the native insulin. The mixed anhydride
reacts with one or more of the A-1, B-1 or B-29 free
15 amino groups on the insulin to form a mono-, di- or
triglycosylated insulin via an amide linkage. The degree
of substitution will depend on the molar ratio of
intermediate to insulin and on reaction condition
including pH. Generally, the molar ratio of intermediate
20 to insulin will vary from 2 to 10. For purposes of
reaction, a pH range of about 8 to 9.5 is preferable.

  Because of the complexity of the reaction, one will
seldom produce the glycosylated insulin as a mono-, di-
or trisubstituted derivative. Rather, a mixture will be
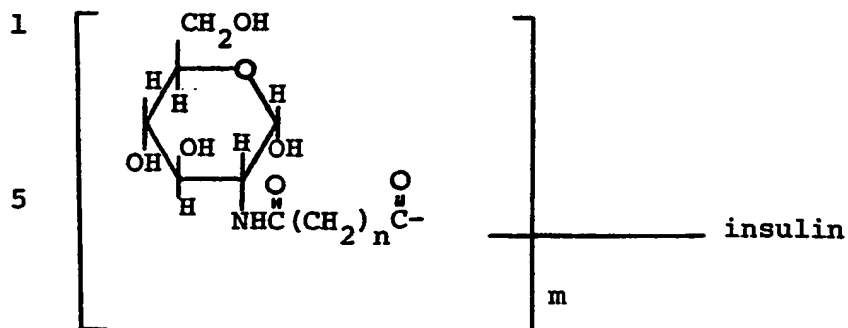25 obtained as shown in the following examples.

  For purposes of description, the glycosylated
insulins may be divided into three categories.

  The first category is those insulins prepared from

30 glucosamines having a $-\overset{O}{\overset{\|}{C}}(CH_2)_n-\overset{O}{\overset{\|}{C}}-$ spacer and possessing
the general formula

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1

$$\left[\begin{array}{c} \text{glycosyl structure with } CH_2OH, H, OH, NHC(CH_2)_nC- \end{array} \right]_m \underline{\hspace{3cm}} \text{insulin}$$

5
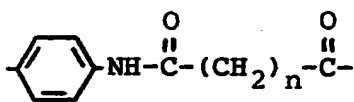
10   wherein n is an integer of 2 to 6, m is an integer of 1
      to 3 and wherein the glycosyl group is attached to the
      insulin through one or more of the α-amino groups of the
      A-1 glycine, B-phenylalanine or ϵ-amino group of the
      B-29 lysine moieties of the insulin molecule.

15   Representative insulins are (glucosamidosuccinyl-)$_m$
      insulin and (glucosamidoglutaryl-)$_m$ insulin.
      A second category encompasses the gluco- and

      mannopyranosides coupled with a $\langle\bigcirc\rangle$-NH-$\overset{O}{\overset{\|}{C}}$-$(CH_2)_n$-$\overset{O}{\overset{\|}{C}}$-

20

      spacer having the general formula

$$\left[\begin{array}{c} \text{glyco structure with } CH_2OH, H, OH, HO, Z, X, O-\langle\bigcirc\rangle-NH-\overset{O}{\overset{\|}{C}}(CH_2)_{\overline{n}}\overset{O}{\overset{\|}{C}} \end{array} \right]_m \underline{\hspace{2cm}} \text{insulin}$$

25
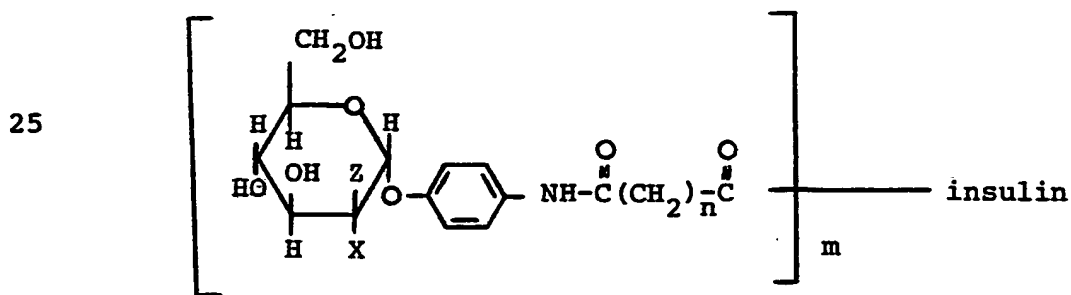
30

      wherein X and Z are different and are selected from the
      group consisting of -H and -OH, n is an integer of 2 to 6
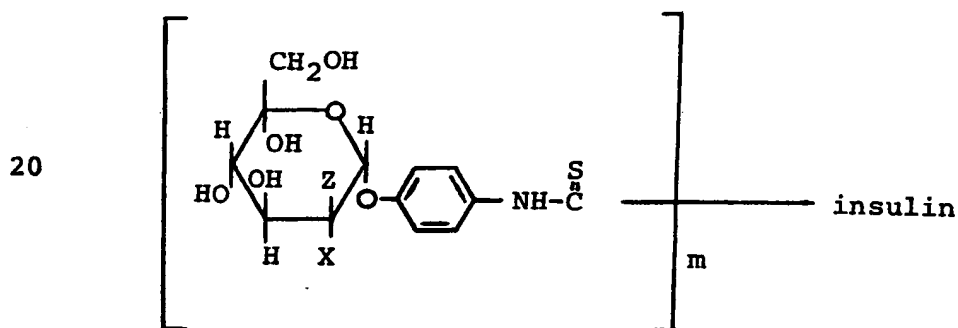
-11-

1   and m is an integer of 1 to 3, and wherein each glycosyl
     group is attached to the insulin by an amide linkage
     through one or more of the α-amino groups of the A-1
     glycine, B-1 phenylalanine or ϵ-amino group of the B-29
5   lysine moieties of the insulin molecule.
         Representative compounds include [p-(α-D-gluco-
     pyranosyloxy)-phenyl-N-succinamyl] insulin;
     [p-(α-D-glucopyranosyloxy)-phenyl-N-glutaramyl]$_m$ insulin;
     [p-(α-D-mannopyranosyloxy)-phenyl-N-succinamyl]$_m$ insulin;
10   and [p(α-D-mannopyranosyloxy)-phenyl-N-glutaramyl]$_m$
     insulin.
         The third category is inclusive of gluco- and

                                                    S
                                                    ‖
     mannopyranosides coupled with a —⟨   ⟩—NHC— spacer having
.15 the general formula

20
$$\left[\begin{array}{c} \text{CH}_2\text{OH} \\ \text{(pyranose ring)} \end{array} \quad \text{O}-\text{(phenyl)}-\text{NH}-\overset{\text{S}}{\underset{\text{‖}}{\text{C}}} \quad \right]_m —— \text{insulin}$$

25   wherein Z and X are different and are selected from the
     group consisting of -H and -OH, and m is an integer of 1
     to 3 and wherein each glycosyl group is attached to the
     insulin by a thioamide linkage through one or more of the
     α-amino groups of the A-1 glycine, B-1 phenylalanine or
30   ϵ-amino groups of the B-29 lysine moieties of the
     insulin molecule.

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1   Representative compounds include [p-α-D-
glucopyranosyloxy)-phenyl-thiocarbamoyl-]$_m$ insulin and
[p-(α-D-mannopyranosyloxy)-phenyl-thiocarbamoyl-]$_m$
insulin.

5   The glycosylated insulins prepared according to this
invention may be administered to a diabetic in any
conventional manner, i.e., subcutaneous, intramuscular or
intraperitoneal injection. The dosage may be the same in
terms of IU (international units) as will free or native

10   insulin. Since dosages vary widely according to the
needs of the patient, no attempt will be made to try to
define dosage ranges. That will be left to the judgment
of the patient's physician. Generally, dosages of 2 mg
of insulin per day are required for a 60 Kg. man.

15   The following examples show the preparation of the
intermediate compounds, the preparation of glycosylated
insulins, their bioactivity and ability to inhibit or
prevent aggregation.
EXAMPLE I:

20   Preparation of N-succinyl glucosamine
         Glucosamine hydrochloride (0.05 $\underline{m}$. 10.78 g) was
dissolved in 15 mls of double distilled water and 0.05
$\underline{m}$ triethylamine (6.95 ml). To this was added, with
stirring, succinic anhydride (0.05 $\underline{m}$, 5.705 g) in 37.5

25   ml of acetone. The resulting mixture separated into two
phases and sufficient water was added to bring both
phases into a single solution. The solution was held at
room temperature for four (4) hours for the reaction to
be completed after which it was placed in a vacuum

30   chamber and evaporated until a viscous, yellowish
concentrated solution was obtained. The concentrate was
measured and diluted with a triple amount of glacial

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1   acetic acid resulting in the formation of a white
     precipitate of N-succinyl glucosamine. The product was
     separated from the acetic acid solution by filtration and
     washed with ethanol and then petroleum ether. The yield

5   of the resulting product was 39%. The product had a
     melting point of 174-175° C and a molecular weight within
     2.5% of the calculated mole weight of 279.26. The
     structure and molecular weight were confirmed by IR, NMR
     and MS/GC spectra.

10   EXAMPLE II:
             Preparation of N-glutaryl glucosamine
         The procedure of Example I was followed using
     glutaric anhydride. The product yield was 41%. The
     melting point was 195-196° C. The calculated mole weight

15   was 293.27. Structure was confirmed by IR and NMR
     spectra.
     EXAMPLE III:
             Preparation of p-(succinylamido)-phenyl-
                   α-D-glucopyranoside

20   In a first step, p-nitrophenyl-α-D glucopyranoside
     (14 m mole, 4.214 g) in 350 ml of methanol was reduced by
     mixing with ammonium formate (56 m mole, 3.54 g) and
     palladium on carbon particles at 25° C. The system was
     flushed for four (4) hours with nitrogen after which it

25   was filtered and the filtrate was evaporated at a reduced
     pressure. The crude p-aminophenyl-α-D-glucopyranoside
     was purified by recrystalization in an ethanol-water
     (50:1) mixture. The yield was 71%. Its melting point was
     169-170° C. Structure and molecular weight were

30   confirmed by IR and MS/GC spectra. The observed
     molecular weight was within 2.7% of the calculated mole
     weight of 271.27.

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1   Following the procedure of Example I, p-aminophenyl-
α-D-glucopyranoside was reacted with succinic anhydride to
produce p-(succinylamido)-phenyl-α-D glucopyranoside in a
yield of 53%. The melting point of the product was
5   178-180° C. Structure and molecular weight were
confirmed by IR, NMR and MS/GC spectra. The observed
molecular weight was within 2% of the calculated mole
weight of 371.34.

EXAMPLE IV:

10   Preparation of p-(glutarylamido)-phenyl-
                      α-D-glucopyranoside

The procedure of Example III was followed using
glutaric anhydride in the place of succinic anhydride.
The p-(glutaryl-amido)-phenyl-α-D glucopyranoside was
15   produced in a yield of 63% and had a melting point of
167-168° C. Structure was confirmed by IR spectra and
the calculated mole weight was 385.37.

EXAMPLE V:

                Preparation of p-(succinylamido)-phenyl-
20   α-D-mannopyranoside

First, p-nitrophenyl-α-D-mannopyranoside was reduced
to p-aminophenyl-α-D-mannopyranoside using the procedure
outlined in Example III. The product yield was 91% and
the product melted at 150-153° C. The structure was
25   verified by IR spectra.

The p-aminophyenyl-α-D-mannopyranoside thus produced
was reacted with succinic anhydride in the manner
described in Example III to produce p-(succinylamido)-
phenyl-α-D-mannopyranoside having a melting point of
30   65-66° C in 67% yield. Structure and molecular weight
were confirmed by IR, NMR and MS/GC spectra. The

-15-

1   observed molecular weight was within 2% of the calculated

molecular weight of 371.34.

EXAMPLE VI:

Preparation of p-(glutarylamido)-phenyl-

5   α-D-mannopyranoside

The procedure outlined in Example V was followed

substituting glutaric anhydride for succinic anhydride.

The resulting p-(glutarylamido)-phenyl-

α-D-mannopyranoside melting at 134-136° C was produced in

10   a yield of 75%. The calculated molecular weight was

385.37. Structure was confirmed by IR spectra.

EXAMPLE VII:

Preparation of p-isothiocyanotophenyl-

α-D-glucopyranoside

15   To a solution of p-(aminophenyl)-α-D-glucopyranoside

in 80% aqueous ethanol was added a molar excess of

thiophosgene ($CSCl_2$). The reaction was carried out at

room temperature and was complete in a manner of minutes.

A crystalline product was obtained. The calculated mole

20   weight was 313.3.

EXAMPLE VIII:

Preparation of p-isothiocyanotophenyl-α-

D-mannopyranoside

The procedure of Example VII may be followed

25   substituting p-(aminophenyl)-α-D-mannopyranoside for the

corresponding glucopyranoside to produce

p-isothiocyanato-phenyl-α-D-mannopyranoside.

In confirming the synthesis of the above

combinations of glucosamine or p-aminophenyl-α-D gluco-

30   and mannopyranosides with succinic and glutaric

anhydrides, the following tests were utilized. An

infrared spectrophometer (Beckman Microlab 620 MX

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1   Computing Infrared Spectrophometer) was utilized to
     determine the reaction between the amino group and the
     anhydride by detecting the presence of an amide bond.
     Samples were prepared as 0.5% (w/w) KBr pellets. The
5   presence of the amino group prior to reaction was
     detected by the N-H bending vibration at 1650-1580 cm^{-1}.
     The p-aminophenyl derivatives prepared by the reduction
     of the corresponding p-nitrophenyl derivatives did not
     show N-O stretching bands at 1580 cm^{-1} and 1330 cm^{-1}
10   indicating that the reduction reaction was complete. The
     formation of the amide bond was shown by the presence of
     a C=O stretching band at 1660 cm^{-1} and a N-H bending mode
     at 1600 cm^{-1}. A normal dimeric carboxylic C=O stretching
     band was also found at about 1725 cm^{-1}. These data
15   confirm a distinct amide band indicating the completion
     of the coupling reaction between the amino and
     dicarboxylic acid anhydride reactants.
         The molecular weights were determined by MS/GC
     spectra using a LKB 9000S MS/GC spectrophometer
20   interfaced with a DEC PDP 11/34 computer. The volatility
     of the carbohydrate derivatives was enhanced by using
     trimethylsilyl derivatization of the hydroxyl and
     carboxylic acid groups. In all instances, the observed
     molecular weight of the trimethylsilyl derivatives was
25   within $2.5^{\pm}$ 0.5 of the calculated theoretical values.

         The presence of the $-NH\overset{O}{\overset{\|}{C}}(CH_2)_2COOH$ moiety was
     confirmed by proton MNR spectra using a JOEL JNM-FX 270
     Fourier Transform NMR spectrophometer. The samples were
30   dissolved in D_2O and sodium 2,2-dimethyl-2-
     silapentane-5-sulfonate (DSS) was used as an internal
     reference. For example, in the p-(succinylamido-

-17-

1   α-D-glucopyranoside, the proton signals of the methylene
     groups in the succinyl moiety was observed at δ=2.71 as
     a triplet. the peak area was proportional to the number
     of protons representing the four methylene protons of the
5   succinyl moiety.

          The melting points were determined by the capillary
     melting point method.

          The yield of the above glucose and mannose
     derivatives with the dicarborylic acid anhydrides varied
10   between about 39 and 91%. The variation in yield is
     thought to be due to the use of a limited solvent
     (ethanol-water mixture) for recrystallization. The yield
     should increase with the selection of a proper solvent
     for the recrystallization procedure.

15   The recoupling action of the above described
     glycosylamidocarboxylic acid derivatives with insulin is
     carried out via a mixed anhydride method wherein the
     mixed anhydride is not isolated from the reaction
     mixture. The glycosylamidocarboxylic acid is converted
20   to mixed anhydride by reaction with isobutylchloroformate
     and the resulting mixed anhydride is reacted with a free
     amino group from the insulin molecule to form an amide
     linkage. The procedure is described in general by
     Erlanger et al in J. Biol. Chem., 228,713 (1957) and
25   Arekatsu et al in J. Immunal., 97, 858 (1966).

          There are three primary sites available on the
     insulin molecule for reaction with the glycosylamido-
     carboxylic acid derivatives and the insulin may be
     coupled with one, two or three of these derivatives.
30   These available sites include the α-amino groups of the
     glycine (Gly A-1), and phenylalanine (Phe B-1) and the
     ϵ-amino group of the lysine (Lys B-29) portions of the

-18-

1   insulin molecule. The pKapp values of these groups are:
      8.0 for Gly A-1, 6.7 for Phe B-1 and 11.2 for Lys B-29.
           Because insulin becomes denatured at too high a pH
      and to maintain the ϵ-amino group of the Lys B-29 moiety
5   in a less reactive protonated state, the pH of the
      coupling reaction between the glycosyl-amido-carboxylic
      acids and insulin was chosen to be between 7.5 and 10 and
      preferably at 9.5. Therefore, the α-amino groups of the
      Gly A-1 and Phe B-1 positions are thought to be the
10   primary reaction sites. However, trisubstituted
      glycosylated insulin may also be produced by the above
      method since a free ϵ-amino group from the Lys B-29
      moiety could be formed by deprotonation through the use
      of the highly nucleophilic tri-N-butylamine added to
15   complex the HCl produced during the anhydride formation
      by the isobutylchloroformate. Also, based on the
      Henderson-Hasselbach equation, at a pH of 9.5, about 2%
      of the ϵ-amino groups of Lsy B-29 exist in equilibrium in
      the free or deprotonated form. Therefore, a significant
20   amount of trisubstituted glycosylated insulin may be
      prepared. However, because of the pH chosen, i.e., 9.5
      and the more reactive free amino groups of Gly A-1 and
      Phe B-1 at that pH, the glycosylated insulin will be
      primarily a mixture of di and tri-substituted
25   derivatives. Some monosubstitution may also be present.
           In the following examples, the unreacted insulin is
      removed from the glycosylated insulin by means of
      affinity chromatography using a column containing
      Sepharose beads bound with Con-A (Concanavalin-A).
30   It is known that Con-A has a binding affinity for
      saccharides. Therefore, the more glycosyl moieties
      coupled to the insulin, the greater that glycosylated

-19-

1   insulin will be bound to the Con-A in the chromatography
     column. One would then expect the unreacted insulin to
     be eluted through the column first followed by mono-, di-
     and tri-glycosylated insulins in that order.

5   This is generally true. However, some glycosylated
     derivatives may be eluted from the column along with
     unreacted insulin.

          The following example is typical of the process of
     separating unreacted insulin from glycosylated insulin by

10   affinity chromatography with Con-A.

     EXAMPLE IX:

                   Preparation of N-succinylglucosamine
              Coupled Insulin (Glucosaminosuccinyl Insulin)

               Bovine insulin (87.77 μ moles 500 mg) was dissolved

15   in 200 mls of an equal volume mixture of distilled water
     and dimethylformamide (DMF) and adjusted to a pH of 9.5
     with 0.1 N sodium hydroxide and was then cooled in an ice
     bath. N-succinylglucosamine, (800 μ moles) was dissolved
     in a solution of DMF containing 800 μ moles each of

20   tri-N-butylamine and isobutylchloroformate and kept at 0°.
     C for 20 minutes. An additional 1.6 m mole of
     tri-N-butylamine was added to this solution which was
     then mixed, with stirring, to the insulin solution. The
     reaction mixture thus formed was pH adjusted to 9.5 with

25   0.1 N sodium hydroxide and kept for one hour at 0° C.
     The mixture was then kept at room temperature overnight
     and then dialyzed through a semipermeable membrane for
     two days against distilled water to remove unreacted
     N-succinylglucosamine. The distilled water was

30   maintained at 4° C and was changed every four hours.

          The glycosylated insulin remaining inside the
     dialysis membrane was lyophilized and dissolved in the

-20-

1   tris-buffer solution described below. The resulting
     solution was sterilized by filtration to remove any
     bacteria present.

          The sterilized product was placed on a 2.5 X 60 cm
5   column containing beads of commercial Con A
     (Concanavalin-A) bound to Sepharose 4B (Sigma Chemical
     Co., St. Louis, Mo.). The unreacted insulin was removed
     from the column using a 0.02 $\underline{m}$ tris-buffer eluent also
     containing $1\underline{mm}$ $MnCl_2$, $1\underline{mm}$ $CaCl_2$ and 0.5 $\underline{m}$ NaCl.
10   The eluent had a pH of 7.4 and was maintained at 4° C.
     The flow rate was maintained at 72 ml/hr and 7.0 ml
     fractions were collected and analyzed by UV spectra at A
     276 nm for the presence of insulin. A colorimetric
     determination for sugars at 480 nm using a
15   phenol-sulfuric test also showed the presence of some
     N-succinylglucosamine coupled insulin.

          After approximately 105 minutes as shown by FIG. 1,
     all of the unreacted insulin (component 1) had been
     collected as monitored by the UV spectra at 276 nm. At
20   that time, $0.1\underline{m}$ α-methyl-D-mannopyranoside was added
     to the tris-buffer solution as an eluent and the flow
     rate was maintained at 72 ml/hr. After approximately 200
     minutes, all of component 2, consisting of
     N-succinylglucosamine, coupled insulin, had been
25   collected as also shown in FIG. 1.

          The low intrinsic binding capacity of the
     glucosamine moiety to Con-A was thought to be responsible
     for the mixed elution of free insulin and glycosylated
     insulin in component 1. Due to the low absorptivity of
30   the glycosylated insulin in Component 2 at 480 nm, the
     degree of substitution could not be determined.

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1   The glycosylated insulin in component 2 was
        lyophilized for determination of its ability to depress
        blood sugar levels.
            The corresponding N-glutarylglucosamine coupled
5   insulin (glucosaminoglutaryl insulin) was prepared in a
        similar manner.

EXAMPLE X:

                Preparation of p-(succinylamido)-phenyl-
                α-D-glucopyranoside Coupled Insulin
10   [p-(α-D-glucopyranosyloxy)-phenyl-N-succinamyl insulin]
            The procedure of Example IX was followed for
        reacting the p-(succinylamido)-phenyl-α-D-glucopyranoside
        from Example III with bovine insulin. The results are
        shown in FIG. 2. Component 1 in FIG. 2 consisted of free
15   insulin and some glycosylated insulin as verified by the
        phenol-sulfuric acid method at 480 nm. Components 2 and
        3 were collected and tested by the phenol-sulfuric acid
        method for the presence of the glycosyl radical as well
        as at 276 nm for insulin. Due to the large amount of
20   eluent required to separate component 3, it can be
        predicted that Component 3 contained more glycosyl
        radicals on the insulin than Component 2. The area under
        the curves of Components 2 and 3 was 58.9% and 41.1%
        respectively. Component 2 was primarily diglycosyl
25   substituted insulin and Component 3 was primarily the
        triglycosyl substituted derivative. Therefore, 0.589 X 2
        + 0.411 X 3 = 2.411 which would be the average number of
        glycosyl derivatives on the insulin contained in
        Components 2 and 3 combined. This degree of substitution
30   was consistent with the phenol-sulfuric acid test which
        showed 2.3 glycosyl groups per insulin molecule. The

1   phenol-sulfuric acid test is detailed by Dubois et al,

        Analytical Chemistry, 28, 350 (1956).

           After collection Components 2 and 3 were combined

        and dialyzed to remove the eluent, α-methyl-D-

5   mannopyranoside, the purified product was lyophilized for

        biological testing.

           Following the same procedure, the corresponding

        p-(glutarylamido)-phenyl-α-D-glucopyranoside coupled

        insulin [p-α-D-glucopyranosyloxy)-phenyl-N-glutaramyl

10   insulin] was prepared.

        EXAMPLE XI:

             Preparation of p-(succinylamido)-phenyl-

                 α-D-mannopyranoside Coupled Insulin

         [p-(α-D-mannopyranosyloxy)-phenyl-N-succinamyl insulin]

15   The procedure outlined in Example X was followed and

        the elution profile is shown in FIG. 3. Component 1 was

        unreacted free insulin since the phenol-sulfuric acid

        test was negative. The average degree of glycosyl

        radicals attached to insulin for the combination of

20   components 2 and 3 was 2.5 according to the

        phenol-sulfuric acid test. The area under the curves for

        components 2 and 3 was 34% and 66% respectively

        indicating an average degree of substitution of 2.66

        which compares closely with the above test results.

25   The purified lyophilized product was retained for

        testing for blood sugar reduction.

           The corresponding p-(glutarylamido)-phenyl-α-D-

        mannopyranoside coupled insulin [p-(α-D-mannopy-

        ranosyloxy)-phenyl-N-glutaramyl insulin] was prepared and

30   purified by the above procedures.

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1   EXAMPLE XII:

              Preparation of p-(α-D-glucopyranosyloxy)-
                   phenyl-thiocarbamoyl Insulin

         p-(Isothiocyanotophenyl)-α-D-glucopyranoside (355.08

5   μmoles) from Example VII was dissolved in a solution of
     three parts pyridine and one part water at 5° C and the
     pH was adjusted to 8.0 with 0.1 N NaOH. Bovine insulin
     (177.54 μ moles, 1 gm) was prepared using a
     pyridine-water solvent and combined with the

10   glucopyranoside solution. The combined solutions were
     maintained at 5° C at a pH of 8.0 for one hour and then
     allowed to stand overnight at room temperature. The
     reaction product consisting of p-(α-D-glucopyranosyloxy)
     -phenyl-thiocarbamoyl insulin was then dialyzed as in

15   Example IX to remove unreacted p-(isothiocyanotophenyl)-
     α-D-glucopyranoside and the remaining product was
     lyophilized, dissolved in tris-buffer and subjected to
     affinity chromatography on a Con-A Sephorase 4B column as
     in Example IX. The flow rate was 26 ml/hr at 4° C and

20   5.0 ml fractions were collected. The elution profile is
     shown in FIG. 4. Component 1 contained both free insulin
     and glycosylated insulin and Component 2 consisted of
     p-(α-D-glucopyranosyloxy)-phenyl-thiocarbamoyl insulin
     having an average of 1.5 glycosyl groups per insulin

25   molecule.
         The product from component 2 was dialyzed to remove
     the α-methyl-D-mannopyranoside eluent and was then
     lyophilized for biological testing.
     EXAMPLE XIII:

                        Aggregation Studies

30   One of the problems associated with free or native
     insulin is its tendency to aggregate and eventually
     crystallize out of solution, thereby reducing its
     bioavailability. With the glycosylated insulins this

-24-

1   tendency is greatly reduced since portions of the active
     amino sites on the Gly A-1, Phe B-1 and Lys B-29 in
     insulin are blocked by the coupling reaction of glycosyl
     groups.

5   Bulk aggregation studies with free insulin compared
     with glycosylated insulins were carried out by two
     methods. In a bulk aggregation study, various aqueous
     insulin and glycosylated insulin solutions containing 0.1
     mg/ml of insulin were stirred at 1555 rpm until

10   aggregation was visually observed or up to two weeks. In
     a second test, solutions containing the same insulin
     concentration were deposited on polyurethane (Biomer) and
     microscopically observed for aggregation.
          The results are as follows:

15   TIME REQUIRED FOR AGGREGATION

Aggregation Test	Free Insulin	Glycosylated Insulins		
		A	B	C
Bulk	2 - 3 days	2 weeks	2 weeks	2 weeks
Polyurethane	1 - 2 days	2 weeks	2 weeks	8 days

20   A = p(α-D-glucopyranosyloxy)-phenyl-N-succinamyl insulin
     B = p(α-D-mannopyranosyloxy)-phenyl-N-succinamyl insulin
     C = p(α-D-glucopyranosyloxy)-phenyl-thiocarbamoyl insulin
          It is obvious from the above results that the
     glycosylated insulins are much more stable against

25   aggregation than free insulin and will thus have a better
     storage life.

     EXAMPLE XIV:

30   Bioactivity of Glycosylated Insulins
          The bioactivity of the glycosated insulins described
     herein was determined by a blood sugar depression test

-25-

1 and compared to commercial insulin preparations and
  controls. In this test, replicates of standard
  laboratory rats were fasted for twenty hours. After
  measuring baseline blood sugar levels, a 1 mg/kg dose of
5 either free or glycosylated insulin was injected via an
  intraperitoneal route. The blood sugar level in each rat
  was measured colormetrically 20 minutes after the
  injection. The results are given in the following table:

BLOOD SUGAR DEPRESSION (BSD) TEST

Type of Insulin	No. Rats	Blood Sugar Concentration after 20 min. IP. injection (± S.E.M.) mg/dl
SIGMA INS. (21F-0375) 25.5IU/MG.	5	32.66 ± 1.10
LILLY INS (615-70N-80)100IU/MG.	5	31.87 ± 1.93
Glucosaminosuccinyl insulin (unpruified)	5	37.80 ± 1.13
Glucosaminoslutaryl insulin (unpurified)	4	45.92 ± 3.30
p-(α-D-glucopyranosyloxy)-phenyl-N-succinamyl insulin	5	40.87 ± 1.32
p-(α-D-glucopyranosyloxy)-phenyl-N-glutaramyl insulin	5	40.40 ± 2.53
p-(α-D-mannopyranosyloxy)-phenyl-N-succinamyl insulin	5	44.80 ± 2.37
p-(α-D-mannopyranosyloxy)-phenyl-N-glutaramyl insulin	5	40.60 ± 0.80
p-(α-D-glucopyranosyloxy)-phenyl-Thiocarbamoyl insulin	5	44.67 ± 2.21
Control	*44	64.62 ± 0.61

*Includes all rats used in test at baseline level

-27-

1   It is evident from the above that the seven glycosylated
     insulins prepared, as described herein, all possess
     significant biological activity in depressing blood sugar
     levels.

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CLAIMS

1. A compound of the formula
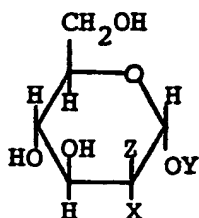
wherein Y is a member selected from the group

consisting of H, $-C_6H_4-NHC(O)-(CH_2)_n-C(O)OH$ or $-C_6H_4-N=C=S$;

X is a member of the group consisting of $-H$, $-OH$

or $-NHC(O)-(CH_2)_n-C(O)OH$; and

Z is a member of the group consisting of $-H$ or $-OH$,
with the proviso that when Y is

$-H$, Z must also be $-H$ and X must be $-NHC(O)-(CH_2)_n-C(O)OH$,

when X is $-OH$, Z must be $-H$ and Y must be

$-C_6H_4-NH-C(O)-(CH_2)_n-C(O)OH$ or $-C_6H_4-N=C=S$, and

when Z is $-OH$, X must be $-H$ and Y must be

$-C_6H_4-NHC(O)(CH_2)C(O)OH$ or $-C_6H_4-N=C=S$;
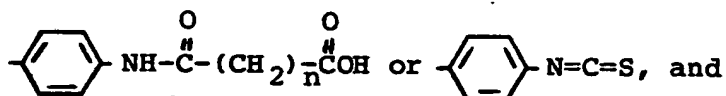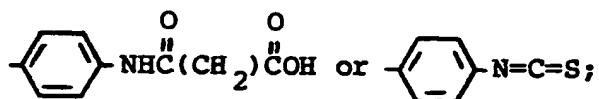
and where n is an integer of 2 to 6.

2. A compound according to Claim 1 wherein Z is

$-H$, Y is $-H$ and X is $-NHC(O)-(CH_2)_n-C(O)OH$; wherein n is an
integer of 2 to 6.

-29-
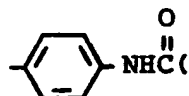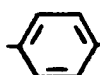
1   3. A compound according to Claim 2 wherein n is 2
and having the formula
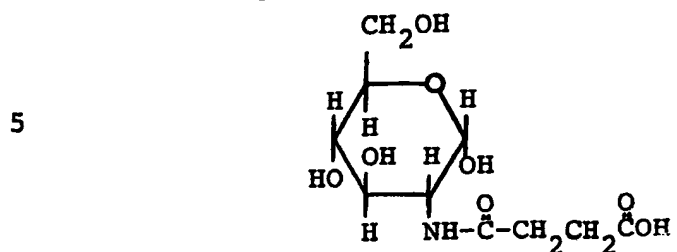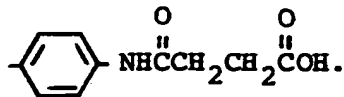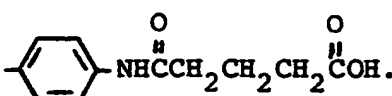
$$CH_2OH$$

5

$$NH-\overset{O}{\overset{\|}{C}}-CH_2CH_2\overset{O}{\overset{\|}{C}}OH$$

4. A compound according to Claim 2 wherein n is 3
10   and having the formula

$$CH_2OH$$
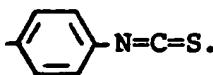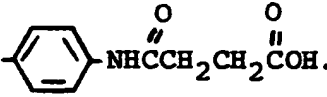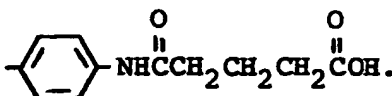
15

$$\underset{\overset{\|}{O}}{N}HC-CH_2CH_2CH_2\underset{\overset{\|}{O}}{C}OH$$

-30-
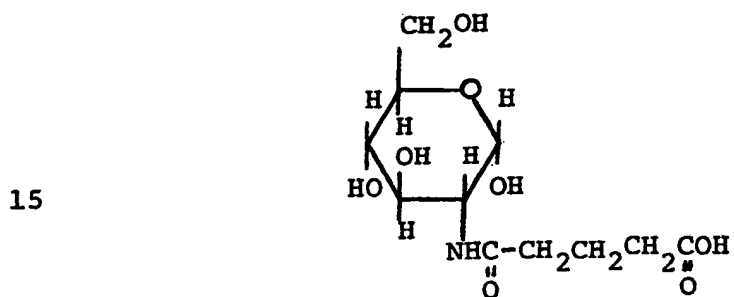
5. A compound according to Claim 1 wherein X and Z are different and are selected from the group consisting

of -H and -OH and Y is a member selected from the group

consisting of ⟨benzene⟩-NHC(CH$_2$)$_{\overline{n}}$COH and ⟨benzene⟩-N=C=S, and n

(with $\overset{O}{\overset{\|}{}}$ and $\overset{O}{\overset{\|}{}}$ above)

is an integer of 2 to 6.

6. A compound according to Claim 5 wherein Z is -H, X is -OH and Y is ⟨benzene⟩-NHCCH$_2$CH$_2$COH.

(with $\overset{O}{\overset{\|}{}}$ and $\overset{O}{\overset{\|}{}}$ above)

7. A compound according to Claim 5 where Z is -H, X is -OH and Y is ⟨benzene⟩-NHCCH$_2$CH$_2$CH$_2$COH.

(with $\overset{O}{\overset{\|}{}}$ and $\overset{O}{\overset{\|}{}}$ above)

8. A compound according to Claim 5 where Z is -H, X is -OH and Y is ⟨benzene⟩-N=C=S.

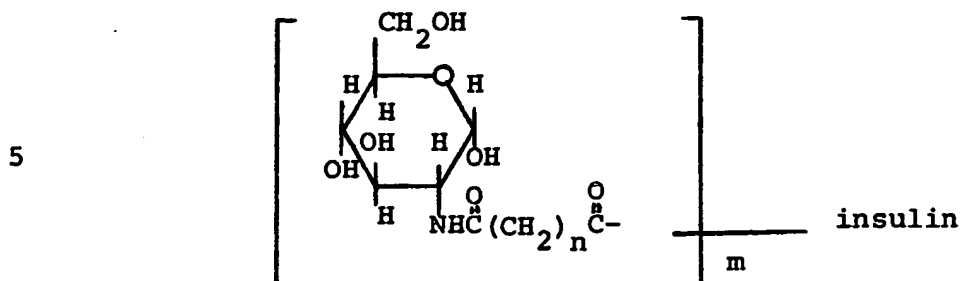9. A compound according to Claim 5 wherein Z is -OH, X is -H and Y is ⟨benzene⟩-NHCCH$_2$CH$_2$COH.

(with $\overset{O}{\overset{\|}{}}$ and $\overset{O}{\overset{\|}{}}$ above)

10. A compound according to Claim 5 wherein Z is -OH, X is -H and Y is ⟨benzene⟩-NHCCH$_2$CH$_2$CH$_2$COH.

(with $\overset{O}{\overset{\|}{}}$ and $\overset{O}{\overset{\|}{}}$ above)

11. A compound according to Claim 5 wherein Z is -OH, X is -H and Y is ⟨benzene⟩-N=C=S.

1   12. A glycosylated insulin having the formula

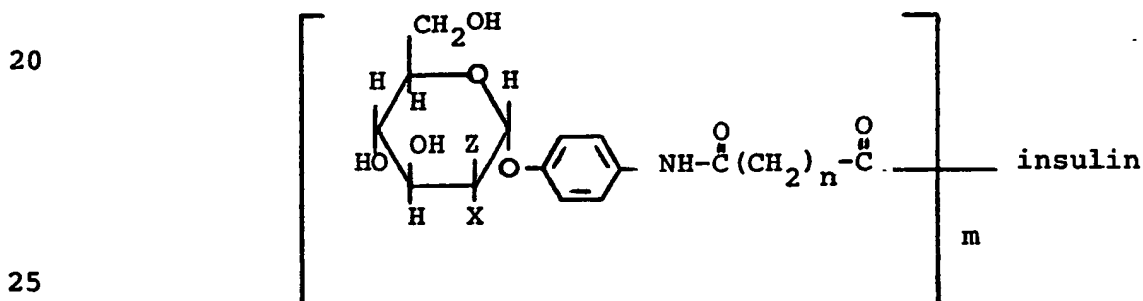5   insulin

wherein n is an integer of 2 to 6, m is an integer of 1
10   to 3 and wherein the glycosyl group is attached to the
insulin through one or more of the α-amino groups of the
A-1 glycine, B-1 phenylalanine or ϵ-amino group of the
B-29 lysine moieties of the insulin molecule.

        13. A glycosylated insulin according to Claim 12
15   wherein n is 2.

        14. A glycosylated insulin according to Claim 12
wherein n is 3

        15. A glycosylated insulin having the formula

20   insulin

25

wherein X and Z are different and are selected from the
group consisting of -H and -OH, n is an integer of 2 to 6
30   and m is an integer of 1 to 3, and wherein each glycosyl
group is attached to the insulin by an amide linkage

-32-

1   through one or more of the α-amino groups of the A-1
     glycine, B-1 phenylalanine or ϵ-amino group of the B-29
     lysine moieties of the insulin molecule.
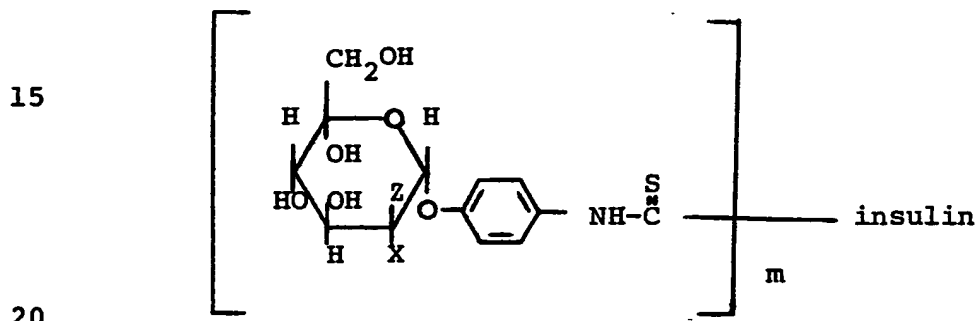
          16. A glycosylated insulin according to Claim 15
5   wherein Z is -H, X is -OH and n is 2.

          17. A glycosylated insulin according to Claim 15
     wherein Z is -H, X is -OH and n is 3.

          18. A glycosylated insulin according to Claim 15
     wherein Z is -OH, X is -H and n is 2.

10   19. A glycosylated insulin according to Claim 15
     wherein Z is -OH, X is -H and n is 3.

          20. A glycosylated insulin having the formula

15

20

          wherein Z and X are different and are selected from the
25   group consisting of -H and -OH, and m is an integer of 1
     to 3 and wherein each glycosyl group is attached to the
     insulin by thioamide linkage through one or more of the
     α-amino groups of the A-1 glycine, B-1 phenylalanine or
     ϵ-amino group of the B-29 lysine moieties of the insulin
30   molecule.
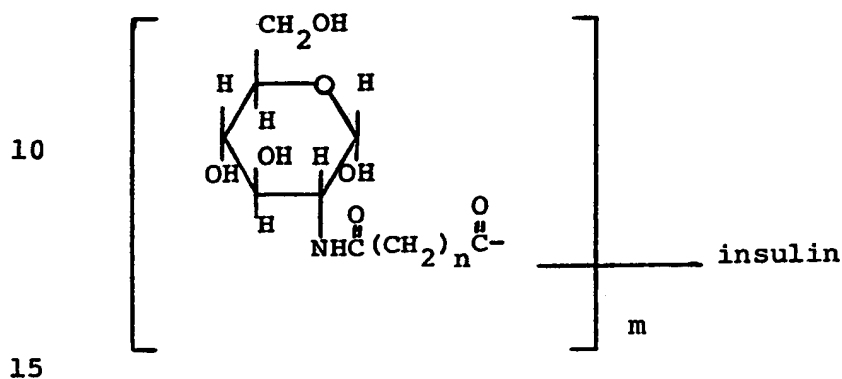
          21. A glycosylated insulin according to Claim 20
     wherein Z is -H and X is -OH.

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1   22.   A glycosylated insulin according to Claim 20
wherein Z is -OH and X is -H.

         23.   A method of depressing the blood sugar level in
a diabetic patient which comprises administering to said
5   patient an effective predetermined amount of a
glycosylated insulin of the formula

$$\left[\begin{array}{c} CH_2OH \\ \end{array}\right]_m \text{---- insulin}$$

15

wherein n is an integer of 2 to 6, m is an integer of 1
to 3 and wherein the glycosyl group is attached to the
insulin by an amide linkage through one or more of the
α-amino groups of the A-1 glycine, B-1 phenylalanine, or
20   ϵ-amino group of the B-29 lysine moieties of the insulin
molecule, in a pharmaceutically acceptable carrier.

         24.   A method according to Claim 23 wherein the
effective amount of the glycosylated insulin has been
determined based upon the needs of the patient.
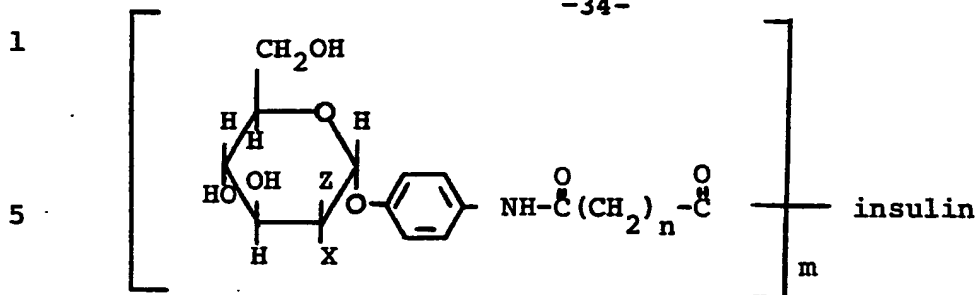25   25.   A method according to Claim 24 where n is 2.

         26.   A method according to Claim 24 where n is 3

         27.   A method of depressing the blood sugar level in
a diabetic patient which comprises administering to said
patient, in a pharmaceutically acceptable carrier, an
30   effective amount of a glycosylated insulin having the
formula

-34-

$$\left[\begin{array}{c} \text{glycosyl-}O\text{-C}_6\text{H}_4\text{-NH-}\overset{\overset{\displaystyle O}{\|}}{C}(CH_2)_n\text{-}\overset{\overset{\displaystyle O}{\|}}{C} \end{array} \right]_m \text{---- insulin}$$

wherein X and Z are different and are selected from the
group consisting of -H and -OH, n is an integer of 2 to 6
and m is an integer of 1 to 3 and wherein each glycosyl
group is attached to the insulin by an amide linkage
through one or more of the α-amino groups of the A-1
glycine, B-1 phenylalamine or ε-amino group of the B-29
lysine moieties of the insulin molecule.   .

28. A method according to Claim 27 wherein the
effective amount of the glycosylated insulin has been
determined based upon the needs of the patient.

29. A method according to Claim 28 wherein Z is H,
X is -OH and n is 3.

30. A method according to Claim 28 wherein Z is -H,
X is -OH and n is 2.

31. A method according to Claim 28 wherein Z is
-OH, X is -H and n is 2.

32. A method according to Claim 28 wherein Z is
-OH, X is -H and n is 3.

33. A method of depressing the blood sugar level in
a diabetic patient which comprises administering to said
patient, in a pharmaceutically acceptable carrier, an
effective amouintof a glycosylated insulin having the
formula

-35-

1

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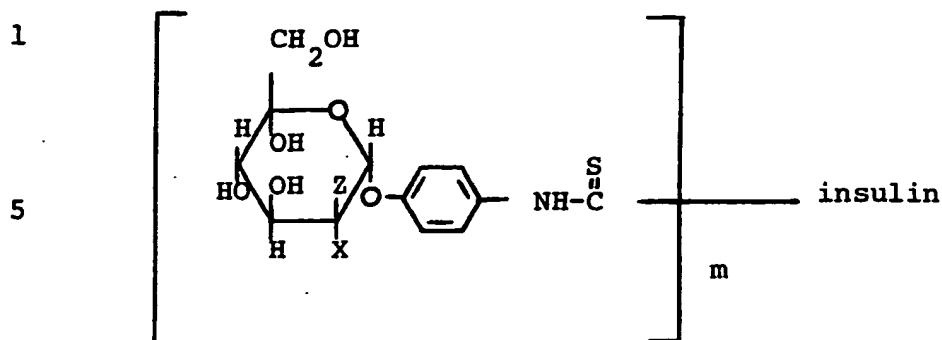
10

wherein Z and X are different and are selected from the
group consisting of -H and -OH, and m is an integer of 1
to 3 and wherein each glycosyl group is attached to the
insulin by a thioamide linkage through one or more of the
15 α-amino groups of the A-1 glycine, B-1 phenylalanine or
ϵ-amino group of the B-29 lysine moieties of the insulin
molecule.

     34.   A method according to Claim 33 wherein the
effective amount of the glycosylated insulin has been
20   determined based upon the needs of the patient.

     35.   A method according to Claim 34 wherein Z is -H
and X is-OH.

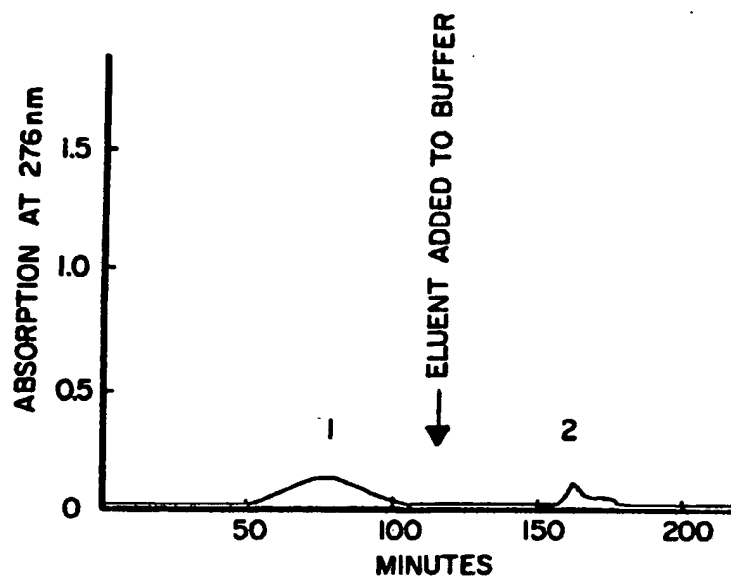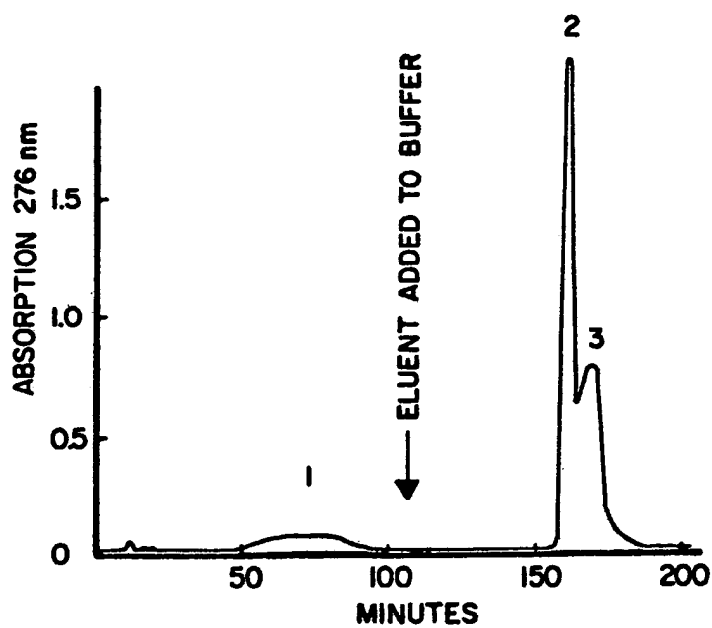   36.   A method according to Claim 34 wherein Z is -OH
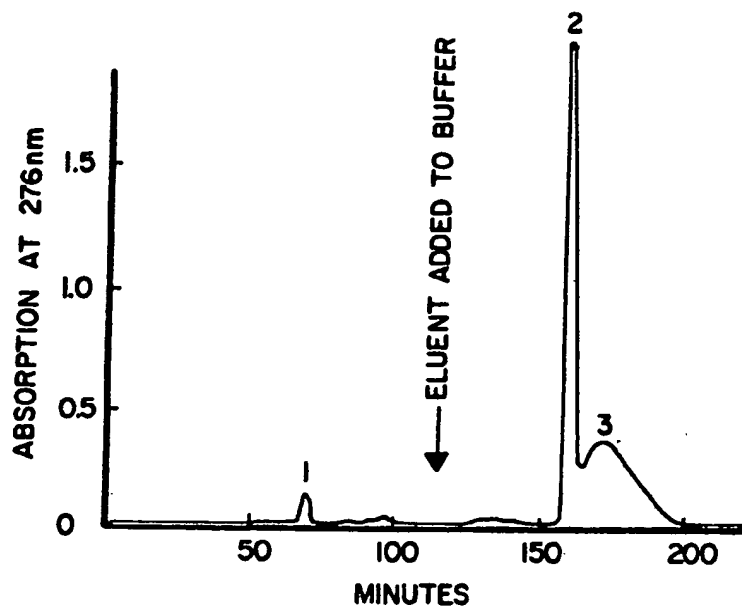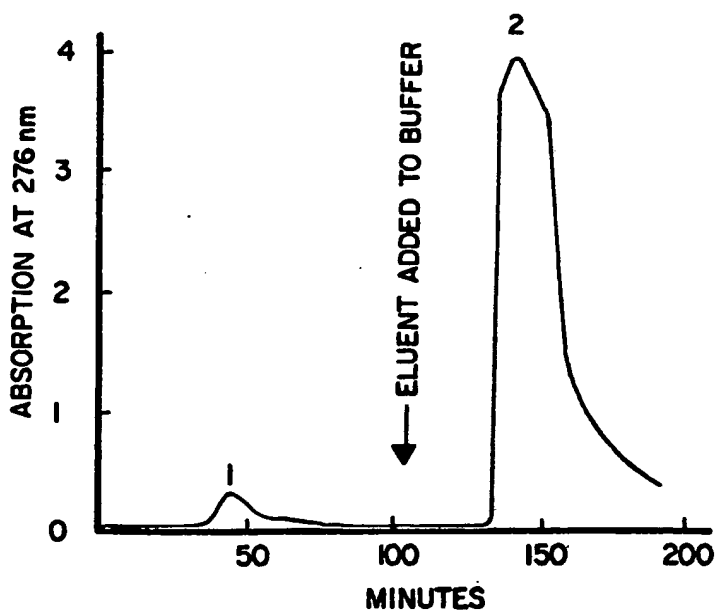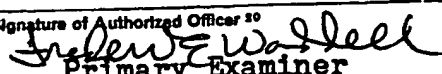and X is -H.

25

Fig. 1

Fig. 2

Fig. 3

Fig. 4

INTERNATIONAL SEARCH REPORT

International Application No PCT/US83/01780

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³

According. 9..ternational Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.³ A61K 37/26; C07C 103/52; C07G 7/00; C07G 11/00;C08B 37/00
U.S. Cl. 424/178; 260/112.7; 536/17.2; 536/17.5; 536/53

II. FIELDS SEARCHED

Minimum Documentation Searched ⁴

Classification System	Classification Symbols
U.S.	424/178; 260/112.7; 536/17.2; 536/17.5; 536/53

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁵

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X	US, A, 4,152,513, published 5 May 1983, AUSTIN ET AL.	1-4
X	US, A, 3,847,890, published 12 November 1974, GREEN	12-36
X	US, A, 3,723,617, published 27 March 1973, SUTTON	1-4
X	US, A, 3,591,574, published 7 July 1971, FENICHEL ET AL.	1-4

* Special categories of cited documents: ¹⁵

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the International filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the International filing date but later than the priority date claimed

"T" later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search ³	Date of Mailing of this International Search Report ²
1 FEBRUARY 1984	14 FEB 1984
International Searching Authority ¹	Signature of Authorized Officer ²⁰
ISA/US	Primary Examiner

Form PCT/ISA/210 (second sheet) (October 1981)